ELSEVIER

Resources, Conservation and Recycling 18 (1996) 135–149

resources, conservation and recycling

Heavy metal tolerance genes: prospective tools for bioremediation

David W. Ow*

Plant Gene Expression Center, Agricultural Research Service, United States Department of Agriculture and the University of California, Berkeley, 800 Buchanan St., Albany, CA 94710, USA

Abstract

Unlike compounds that can be broken down, the remediation of most heavy metals and radionuclides requires removal from contaminated sources. Plants can extract inorganics, but effective phytoextraction requires plants that produce high biomass, grow rapidly and possess high capacity-uptake for the inorganic substrate. Either the existing hyperaccumulator plants must be bred for increased growth and biomass, or that hyperaccumulation traits must be engineered into fast growing, high biomass plants. The latter approach requires fundamental knowledge of the molecular mechanisms in the uptake and storage of inorganics. Much has been learned in recent years on how plants and certain fungi chelate and transport cadmium. This progress has been facilitated by the use of *Schizosaccharomyces pombe* as a model system. As target genes are identified in a model organism, their sequences can be modified for expression in a heterologous host or aid in the search of homologous genes in more complex organisms. Moreover, as plant nutrient uptake is intrinsically linked to the association with rhizospheric fungi, elucidating metal sequestration in this fungus permits additional opportunities for engineering rhizospheric microbes to assist in phytoextraction. Copyright © 1996 Elsevier Science B.V.

Keywords: Cadmium; Phytochelatin; Vacuolar transport; Sulfide; Phytoremediation

* Tel: +510 5595909; fax: +510 5595678.

PII S0921-3449(96)01174-3

1. Introduction

1.1. A role for plants in metal remediation

Global industrialization has been accelerating the release of heavy metals into the environment. Dietary intake of heavy metals through consumption of crop plants can have long-term effects on human health. Unlike organic compounds that can be mineralized, the remediation of inorganics requires removal or conversion into a biologically inert form. Some metals, such as Se and Hg, can be removed through biovolatilization [1–3]. Most other metals, however, requires the physical extraction from soil and water systems. As an alternative to the costly process of conventional engineering efforts, there is increased support for the idea that genetically modified plants can play an important role in extracting heavy metals from contaminated sources [4].

A wide variety of bacterial, fungal, algal and plant systems are capable of concentrating toxic metals from their surroundings. However, no cost-effective way exists to retrieve small organisms from the soil. In contrast, it is possible to harvest contaminant-laden plants, especially if the metals are translocated to shoots. The value of some metals (e.g. Ni, Zn, and Cu) reclaimed from the harvested plant material could provide additional incentive for remediation. Alternatively, the harvested biomass could be disposed of after substantial reduction in volume and weight. The rate of metal removal would depend on the amount of harvested biomass, the number of harvests per year, and the metal concentration in the harvested portion of the plants. Decontaminating a site in a reasonable number of years requires plants with high biomass and high metal accumulation. Plants that exhibit this latter trait do exist and are known as metal-hyperaccumulators [5]. However, they are rather slow growing plants with low biomass and restricted element selectivity. Moreover, little is known of their agronomics, genetics, plant breeding potential and disease spectrum.

The readily harvestable aerial tissues of known hyperaccumulators can concentrate metals ranging from 0.2% (e.g. Cd) to 5% (e.g. Zn, Ni, Mn) of their dry weight [6]. Generally speaking, this is roughly two orders of magnitude higher than the concentrations found in the more familiar crop plants. This comparison assumes that both plant types are capable of growing in the metal contaminated soil, and this is not always the case for most plants that do not tolerate high metal content. Despite this impressive difference in metal uptake, the annual yield in biomass of hyperaccumulators are generally one to two orders of magnitude lower than those of robust crop plants. Thus, if the high yield and hyperaccumulation traits could be bred into a single plant, the effectiveness of metal extraction could increase by one to two orders of magnitude. Plant-based remediation efforts would have a much more practical time-frame, in the order of a decade rather than the current estimates of a century or more.

1.2. Potential for novel metal hyperaccumulation traits

A conventional approach to combining the traits of metal hyperaccumulation and high biomass yield is through breeding programs. However, genetic improvements cannot expand beyond the traits nature provides within sexually compatible species. Modern molecular biology, however, can overcome the sexual barrier through direct gene transfer. In principle, the genetic engineering of novel hyperaccumulation traits directly into fast growing, high-biomass plants should be within the realm of possibility. However, this will require fundamental knowledge of the molecular mechanisms plants use to take up and store toxic ions. In this light, it is important to consider appropriate model systems to expedite basic knowledge in this area.

1.3. Why a fungal model system

Much of recent knowledge about the molecular cellular mechanisms on how plants chelate and store cadmium comes from studies on the fission yeast *Schizosaccharomyces pombe* [7]. Certain fungi share with plants the same cellular response to metal stress. The ease with which fission yeast can be genetically manipulated permits rapid characterization of the molecular processes. Once target genes are cloned from a model organism, their sequences can be modified for gene transfer to a heterologous host, or can aid in the search for homologous genes in more complex organisms. Moreover, as plant nutrient uptake is intrinsically linked to association with rhizospheric fungi, elucidating fungal metal metabolism affords additional opportunities for engineering rhizospheric microbes to assist in plant metal extraction.

The one disadvantage in using a unicellular model system is that it cannot help define multicellular events. Of particular interest would be how plants translocate metals from root to shoot. Nevertheless, an important consideration is that regardless of how plants move metals from one organ to another, the ultimate destination of these ions is to another cell. Therefore, without increasing tolerance and storage capacity at the cellular level, enhancing translocation would merely speed up cellular poisoning in aerial photosynthetic tissue. This effect would, of course, limit biomass and growth rate, the very factors crucial for high capacity metal extraction.

2. Current status of research

2.1. Metal-induced chelators

Intracellular chelation is a well described mechanism in metal tolerance and a key step for metal extraction. In response to heavy metals, animals and certain fungi induce the production of small cysteine-rich proteins known as metallothioneins [8]. The multiple cysteines in metallothioneins chelate metals via formation of thiolate

bonds. There have been numerous reports of engineering the production of animal metallothioneins in plants [9–13]. Varying degrees of increased metal tolerance have been achieved, but none reported substantial increases in overall metal uptake. This may be because plants do not naturally use metallothioneins for metal detoxification. Therefore, the anomalous production of unfamiliar ligands does not lead to further transport and storage of the bound metals. Although metallothionein genes have also been described in plants [14], the proteins have not yet been found, placing uncertainty for their role in metal tolerance. Instead, heavy metals in plants and some fungi induce the production of metal-binding peptides commonly known as phytochelatins [15,16].

S. pombe is one such fungus that shares this response with higher plants. Derived from glutathione (GSH), phytochelatins (PCs) have the general structure of $(\gamma\text{-Glu-Cys})_n\text{Gly}$, where n is generally from 2–5, but as many as 11 γ-Glu-Cys units have been described [17]. Some PC-related peptides lack the carboxyl-terminal Gly or have instead β-Ala, Ser, or Glu. However, these variant peptides are usually found in lower abundance compared to the PCs. As with metallothioneins, the cysteines of PCs form thiolate bonds with the metal cations. A large variety of metals induce the synthesis of PCs, but formation of a PC metal complex has largely been examined with Cd^{2+} and Cu^{2+}. Several reports show that PCs also form complexes with Ag^{+}, Hg^{2+}, Pb^{2+} and Zn^{2+} [18–21]. In the case of Cd^{2+}, two PC-metal complexes can be isolated from cells exposed to Cd^{2+}: a low molecular weight (LMW) PC-Cd complex and a more stable high molecular weight (HMW) PC-CdS complex that contains acid-labile sulfide (S^{2-}). The appearance and the location of the two complexes suggests that the PC-Cd complex acts as a cytoplasmic scavenger and carrier of metals to the vacuole, where it is stored as a stable sulfide-rich chelate.

We have isolated a number of *S. pombe* mutants impaired in metal detoxification. These mutants are hypersensitive to Cd^{2+} and fail to form wild-type levels of one or both of the PC-bound Cd complexes. The sections that follow summarize our current understanding of the molecular biology of cadmium detoxification.

2.2. Vacuolar storage

Through the analysis of LK100, a mutant that fails to form the HMW PC-CdS complex, we isolated a gene encoding a vacuolar membrane protein that is a member of the ATP-binding cassette (ABC) type transporter family [22]. This gene was named *hmt1* (for heavy metal tolerance). We found that the HMT1 protein transports cytoplasmic PC-Cd complex into the vacuole [23]. Table 1 summarized the ATP-dependent transport activities of vacuolar vesicles from HMT1-proficient and deficient strains. ATP-dependent uptake of [^{35}S]-labelled PC-Cd was seen with vesicles from the $HMT1^{+}$, but not the HMT^{-} strain. Only low activity was observed with PC-CdS as a substrate, most likely representing the transport of PC-Cd derived from spontaneous disassociation of the HMW complex. Apo-PC peptides were efficiently transported, however, it remains possible that this activity represent the prior complexation of apo-peptides with traces metals in the vacuolar

vesicle preparation, and a high concentration of Mg^{2+} was provided for the ATP-dependent reaction. Transport of PC-Cd is independent of the pH gradient as it was unaffected by the vacuolar ATPase inhibitor bafilomycin or the H^+-K^+ ionophore nigericin. It was reduced significantly, however, by antibodies directed against HMT1, and by vanadate, an inhibitor known to affect many ABC-type transporters.

When ^{109}Cd-labelled PC-Cd was provided as the substrate, uptake of the label was independent of HMT1. Further examination showed that uptake of the $^{109}Cd^{2+}$ cation is itself independent of HMT1 and vanadate, but is abolished by the inhibitors bafilomycin and nigericin that dissipate the pH gradient. This suggests the presence of an antiport which can use Cd^{2+} as a substrate. Hence, the activity observed with ^{109}Cd-labelled PC-Cd was probably due to free Cd^{2+} disassociated from the complex. In the presence of bafilomycin that blocked the antiport activity, uptake of the label from ^{109}Cd-labelled PC-Cd was dependent on HMT1, and was significantly reduced by the presence of both bafilomycin and vandadate. The data are consistent with the interpretation that HMT1 co-transports PC peptides and Cd^{2+} as a complex.

Higher plants have also been found to transport PC-Cd to the vacuole through what appears to be an ABC-type transporter [24], but the plant transporter gene has not yet been cloned. When the HMT1 protein was overproduced in *S. pombe*, the cells showed enhanced cadmium accumulation and tolerance, presumably due

Table 1
ATP-dependent uptake by vacuolar vesicles

Substrate	Inhibitor	HMT1$^+$	HMT1$^-$
[^{35}S]PC-CdS		−	−
[^{35}S]PC-Cd		+	−
	Bafilomycin	+	−
	Nigericin	+	−
	Vanadate	−	−
	Antibody	−	−
[^{35}S]Apo-PC		+?	−
[^{109}Cd]PC-Cd		+	+
	Bafilomycin	+	−
	Bafilomycin and vanadate	−	−
$^{109}Cd^{2+}$		+	+
	Bafilomycin	−	−
	Nigericin	−	−
	Vanadate	+	+

Vacuolar vesicles were prepared from HMT$^+$ or HMT$^-$ strains. Presence (+) or absence (−) of ATP-dependent transport activity was determined by uptake of radiolabelled substrates in the presence or absence of indicated inhibitors. Adapted from Ortiz et al. [23].
?Indicates uncertainty with transport of apo-PC peptides as complex formation with trace metals has not been ruled out.

to more effective sequestering of the metal. Taken together, these findings suggest that hyperproduction of a PC transporter in the plant vacuolar membrane might similarly enhance the vacuolar sequestration of toxic metals, and thereby enhance greater metal-extraction from contaminated sources [7]. Aside from the ABC-type pump, the activity of a Cd^{2+} antiport has also been reported in plants [25]. As the HMW complex incorporates additional Cd^{2+} ions, it is believed that the additional ions are transported into the vacuole through this route (Fig. 1).

2.3. A cadmium-inducible sulfide-generating pathway

The stable HMW complex found in the vacuole has sulfide ions incorporated into the PC-Cd complex. In the absence of sulfide, the Cys:Cd ratio has been estimated to be from 2:1 to 4:1 [26]. Upon sulfide addition, the Cys:Cd ratio drops to $\sim$ 1:1. Thus, the incorporation of sulfide increases the Cd storage capacity per PC peptide. Furthermore, the HMW complex, which appears to be CdS crystallites surrounded by PC peptides [27], is more stable than the LMW complex in acidic environment [28], such as in the lumen of the vacuole. As formation of the PC-CdS complex is crucial to metal storage, the source of the sulfide ion is an important issue to address. Under metal stress, cellular sulfide levels increase some 7-fold when the cell are grown in rich medium (and higher when grown in minimal medium). Since plants and microbes assimilate inorganic sulfur through a pathway in which sulfate (SO_4^{2-}) is incorporated into ATP and subsequently reduced to form sulfite (SO_3^{2-}) and then sulfide (Fig. 1), a possibility is that the increased sulfide production is due to increased activity of this assimilatory reduction pathway. However, the analysis of one particular mutant, LK69, revealed that Cd-inducible sulfide production requires genes in the purine biosynthetic pathway [29,30].

Fig. 2 shows the relevant segment of the purine biosynthesis pathway (abbreviations defined in the legend). LK69, which is Cd-hypersensitive and deficient in accumulation of PC-CdS complex, was found to harbor genetic lesions in both *ade2* (encoding AMP-S synthetase) and *ade6* (encoding AIR carboxylase). This led to the discovery that an *ade2*$^-$ *ade7*$^-$ double mutant also exhibited the same phenotype as LK69. However, a single lesion in either *ade2*, *ade6* or *ade7* does not cause Cd hypersensitivity. Although AIR carboxylase acts at a step upstream of the AMP-S synthetase reaction, a mutation blocking IMP production via this linear pathway does not exert an epistatic effect on AMP-S synthetase because adenine can be converted to IMP via a salvage pathway. Hence, the IMP to AMP reactions are operational in an *ade6*$^-$ or *ade7*$^-$ mutant grown with adenine supplementation, just as the reactions leading to IMP production catalyzed by AIR carboxylase are operational in an *ade2*$^-$ background. If each segment of the pathway catalyzes a reaction that can be complemented by the other, then blockage of both segments of the pathway would be needed to produce a deficiency phenotype, in this case Cd hypersensitivity.

The reactions performed by the two segments are indeed similar. The conversion of CAIR to SAICAR is analogous to the conversion of IMP to AMP-S in that both reactions incorporate aspartate onto a nucleotide substrate. It has been reported

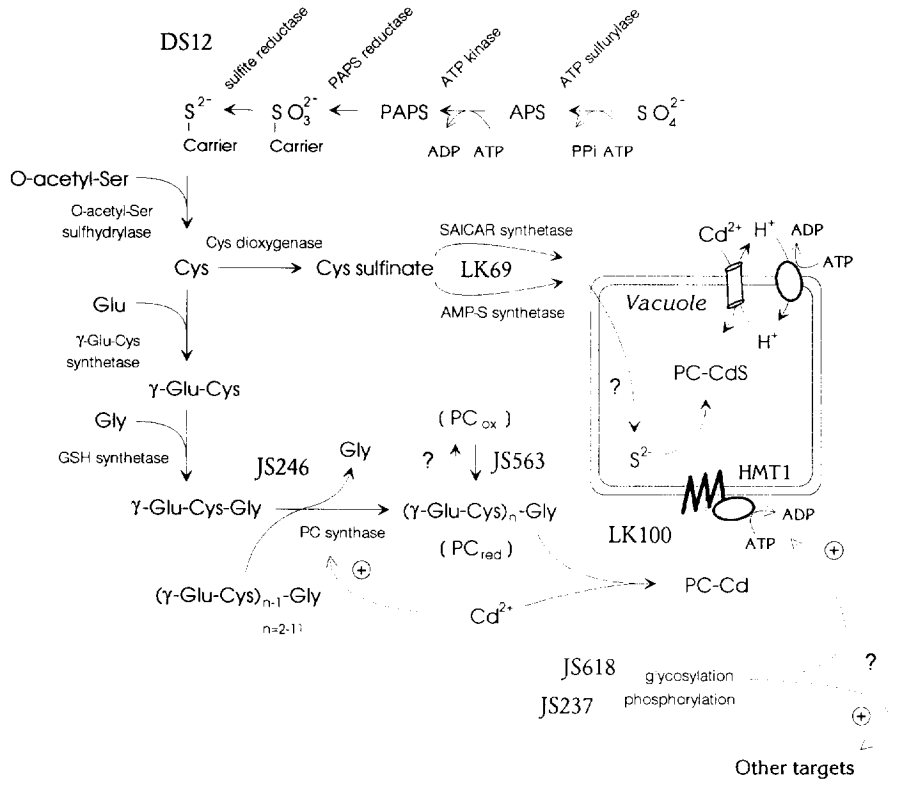

Fig. 1. Molecular model of cadmium detoxification. Cd^{2+} activates the enzymatic synthesis of PC peptides. The peptides are kept from being oxidized by a PC reductase (revealed by mutant JS563). The reduced PC peptides chelate Cd^{2+} and the absence of free Cd^{2+} terminates enzymatic synthesis. The PC-Cd complex, with a Cys/Cd^{2+} ratio from 2:1 to 4:1, are transported into the vacuole by the HMT1 protein (revealed by mutant LK100). S^{2-} and additional Cd^{2+} are added to form a more acid-stable HMW complex, with a Cys/Cd^{2+} ratio of approximately 1:1. Most likely, the additional Cd^{2+} ions enter through a cation antiport, requiring the pH gradient generated by a vacuolar H^+-ATPase. The source of S^{2-}, which is Cd inducible, is not from the SO_4^{2-} assimilatory sulfate reduction pathway (reveal by mutant DS12), but through a novel pathway derived from cysteine and requires enzymes that participate in purine biosynthesis (revealed by mutant LK69). The proposed route starts with formation of Cys sulfinate from Cys, followed by incorporation of Cys sulfinate into purine precursors by SAICAR synthetase and AMP-S synthetase. HMT1 and possibly other proteins involved in metal stress may be affected by glycosylation (revealed by mutant JS618) and phosphorylation (revealed by mutant JS237). Cd stress appears to involve a signal transduction pathway that utilizes cAMP and Ca^{2+} as second messengers. Abbreviations used: APS, adenosine 5′-phosphosulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; AMP-S and SAICAR, see Fig. 2 legend.

that cysteine sulfinate, an oxidative product of cysteine, can replace aspartate in an in vitro reaction catalyzed by the AMP-S synthetase from *Azotobacter vinelandii* [31]. If this analog substrate is also utilized by SAICAR synthetase, then it may be possible that the sulfur-analog intermediates from either segment of the pathway

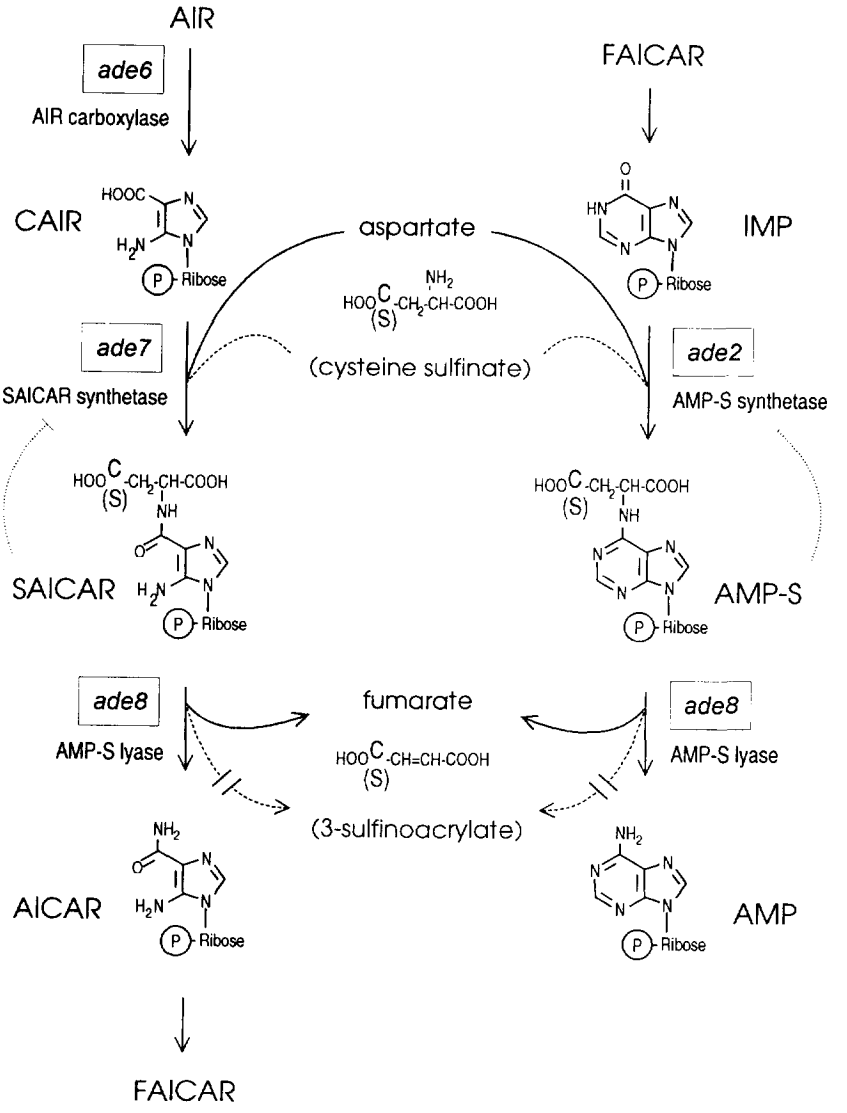

Fig. 2. Relevant segment of the purine biosynthesis pathway and the proposed biosynthesis of cysteine sulfinate derived products (dashed lines). The pathway from AIR to AMP is shown along with hypothetical sulfur analog products (in parentheses). Formation of S-derivatives by SAICAR synthetase and AMP-S synthetase have been shown in vitro. However, AMP-S lyase does not react with these S-derivatives. We propose that AMP-S lyase activity is needed to prevent product inhibition (dotted lines) of SAICAR synthetase and AMP-S synthetase, rather than for the formation of 3-sulfinoacrylate. Abbreviations used: AIR, aminoimidazole ribonucleotide; CAIR, carboxyaminoimidazole ribonucleotide; SAICAR, succinoaminoimidazole carboxamide ribonucleotide; AICAR, aminoimidazolecarboxamide ribonucleotide; FAICAR, formamidoimidazolecarboxamide ribonucleotide; IMP, inosine monophosphate; AMP-S, adenylosuccinate; AMP, adenosine monophosphate. Adapted from Speiser et al. [29] and Juang et al. [30].

can serve in an essential role for Cd tolerance, such as sulfide carriers or donors. Additionally, AMP-S lyase might catalyze the release of the S-analog of fumarate, 3-sulfinoacrylate, as an intermediate in the formation of sulfide and the HMW PC-CdS complex. An *ade8*$^-$ mutant is indeed sensitive to Cd, but exhibits only a slower rate PC-CdS complex formation. To account for the *ade8*$^-$ phenotype, one possibility would be that AMP-S lyase performs a function essential to the biogenesis of the PC-CdS complex, and the slow rate of HMW complex formation could be due to leaky synthesis of active enzyme. An alternative explanation would be that a lack of AMP-S lyase leads to hyperaccumulation of AMP-S and SAICAR, which could feedback inhibit AMP-S synthetase and SAICAR synthetase, respectively. In this latter scenario, a lesion in *ade8* would mimic double lesions of *ade2* and *ade7*. Assuming that product inhibition of enzyme function is less effective than genetic blockage of enzyme synthesis, this could account for the 'leaky' accumulation of the HMW complex seen in the AMP-S lyase-deficient mutant.

In testing the above hypothesis, we examined the activities of AMP-S synthetase, SAICAR synthetase, and AMP-S lyase. The reaction of AMP-S synthetase with [^{35}S]cysteine sulfinate generated a novel [^{35}S]-labeled compound detected by thin layer chromatography. Subsequent addition of AMP-S lyase or crude extracts to the reactions failed to produce evidence for the formation of [^{35}S]sulfinoacrylate. Similarly, SAICAR synthetase reacted with [^{35}S]cysteine sulfinate also formed a novel radiolabeled compound that failed to react further upon addition of AMP-S lyase or crude extracts. Thus, the genetic and biochemical data are consistent with a model that AMP-S synthetase and SAICAR synthetase can incorporate a sulfur analogue of aspartate onto purine intermediates. Sulfur addition to a purine molecule is not without precedent, as assimilation of sulfate begins through incorporation into ATP to form adenosine 5'-phosphosulfate (APS, Fig. 1).

2.4. Sulfide production induced by Pb, but not Cd, requires assimilatory sulfate reduction

The above proposal of a novel Cd-inducible pathway for sulfide production is consistent with the behavior of a mutant blocked in assimilatory sulfate reduction. This mutant, DS12, harbors a lesion in the sulfite reductase gene (Fig. 1) and fails to convert sulfite to the sulfide needed for Cys biosynthesis. As with all Cys auxotrophs, DS12 is hypersensitive to Cd and fails to produce PCs, despite Cys supplementation. Presumably, the slow rate of Cys transport cannot provide the high Cys level for PC production. Upon Cys supplementation, however, the mutant can nonetheless respond to Cd-induced production of sulfide (Fig. 3). This supports our view that sulfide induction during Cd stress can proceed through a pathway starting with Cys as the source. Sulfide production can also be induced by Pb, leading to PbS precipitates. However, this Pb-induced sulfide production is abolished in DS12 (Fig. 3). It thus appears that while the assimilatory sulfate reduction pathway is not needed for Cd-induced sulfide production, it is nonetheless required in the case of Pb.

2.5. FAD/NAD-linked disulfide reductase required for PC accumulation

JS563 was isolated as a mutant defective in the production of Cd-bound PC complexes (Fig. 1). Interestingly, this Cd-hypersensitive mutant also hyperproduces sulfide. The gene responsible for this phenotype was cloned by genetic complementation and was found to restore to JS563 a wild-type level of Cd tolerance, normal accumulation of Cd-bound PC complexes, and normal production of sulfide. In contrast, the cloned mutant allele complemented none of the above defects and was found to harbor a single base change at what appears to be a conserved domain in the encoded protein. The encoded protein shares sequence similarity with members of the FAD/NAD-linked disulfide reductase family. These proteins catalyze the reduction of oxidized substrates, such as cytochrome or glutathione. There is sequence similarity with glutathione reductase (29% similar); however, because the mutant produces adequate amounts of reduced glutathione and because there are some critical differences in the arrangement of the predicted functional sites (redox active cysteines), we do not think that this protein reduces glutathione. Our current hypothesis that it might be a phytochelatin reductase. If PC peptides become oxidized, formation of intra- and inter-molecular disulfide bridges would prevent the sulfhydryl groups from coordinating with Cd^{2+}. Having a dedicated enzyme to reduce intra- and inter-peptide disulfide bridges, similar to the action of GSH reductase for glutathione, would be a plausible solution. In the mutant, the inability to form reduced PC peptides would account for the lack of bound Cd complexes. As PC peptide production is regulated by feedback control, i.e. free metal ions induce enzymatic synthesis, the inability of oxidized PC peptides to bind metals would cause further synthesis of PC peptides. Degradation of large amounts of oxidized PC peptides could account for the aberrantly high sulfide level found in the mutant.

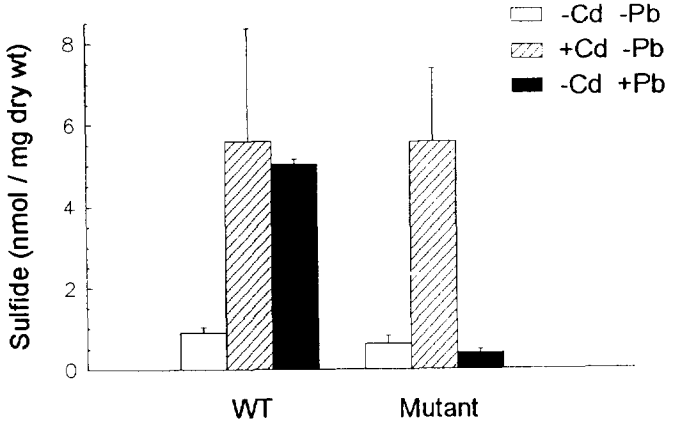

Fig. 3. Direct need for assimilatory sulfate reduction for Pb-induced, but not Cd-induced sulfide production. A sulfite-reductase mutant, auxotrophic for cysteine, can produce Cd-induced levels of sulfide during Cd stress (+ Cd) when provided with cysteine. During Pb stress (+ Pb), however, the mutant is defective in Pb-induced sulfide production.

2.6. Mannose-1-phosphate guanyltransferase required for accumulation of the PC-CdS complex

Recent work on JS618, a mutant unable to accumulate the HMW PC-CdS complex, resulted in the isolation of a gene whose protein product has sequence similarity with mannose-1-phosphate guanyltransferase, an enzyme that converts mannose-1-phosphate to GDP-mannose. GDP-mannose is a substrate for glycosyltransferases in protein glycosylation and polysaccharide biosynthesis. Our current thinking is that the mutation abolishes the glycosylation of a critical protein needed for formation of the HMW complex. As some ABC-type transporters (e.g. the cystic fibrosis transmembrane conductance regulator and the multidrug resistance pump) are glycoproteins, it may be possible that this mutation prevents HMT1 targeting or activity.

2.7. Signal transduction in metal accumulation

The analysis of mutant JS237 suggests that signal transduction involving cAMP and Ca^{2+} is needed for vacuolar accumulation of cadmium. This Cd hypersensitive mutant fails to accumulate the HMW complex. The complementing DNA was found to contain two genes encoding proteins with strong sequence similarities to two human proteins described in recent literature: BTF3 and WASP. Most unusual is that apparently each protein can be encoded on separately transcribed mRNAs or together in one bicistronic mRNA.

BTF3 was initially described to be a basal transcription factor because it co-purified with RNA polymerase II [32]. Recently, it was found that BTF3 is the β subunit of a protein complex known as NAC which binds nascent polypeptide chains as they are formed by the ribosome [33]. NAC competes with the signal recognition particle (SRP). If the nascent chain has a signal sequence, then SRP wins out and the polypeptide is directed to the endoplasmic reticulum (ER). However, if NAC is not present, then SRP will take any polypeptide, even those without the signal sequence, and direct it into the ER pathway. A mutation in NAC would be expected to result in missorting of proteins. In humans, there are at least 9 BTF3 (NAC-β) genes, so it is possible that different NAC complexes direct the transport of different proteins.

WASP was identified as the protein responsible for a chromosome X-linked recessive immunodeficiency disorder known as the Wiskott-Aldrich Syndrome [34]. This Wiskott-Aldrich Syndrome Protein (WASP) was found to interact with the Nck protein though Src homology III domains [35]. Nck is believed to be an adapter protein in signal transduction as it interacts with a protein kinase. This implicates WASP in relating cellular signals, like metal stress, through various protein-protein interactions leading to kinase action. Consistent with this hypothesis, we found that this mutant is also hypersensitive to Ca^{2+}. Ca^{2+} and cAMP are known second messengers in signal transduction and a high Ca^{2+} level reduces the level of cAMP. When cAMP is provided in the medium, the mutant is no longer sensitive to Ca^{2+} or to Cd^{2+}. Since cAMP is known to modulate the activity of

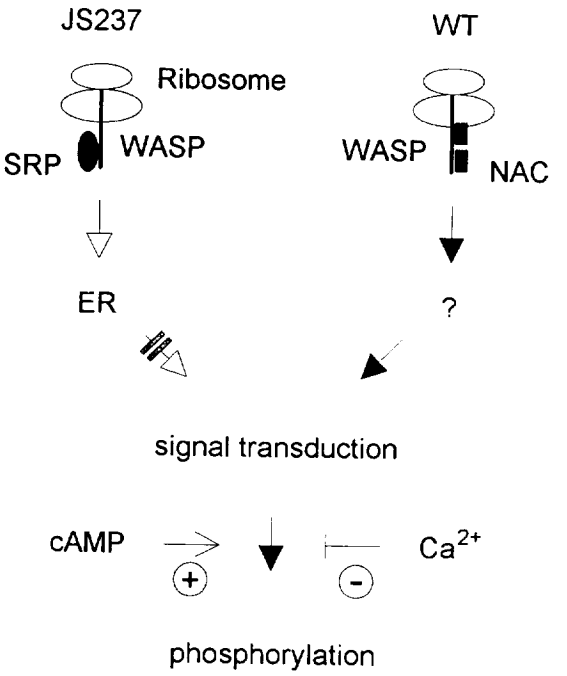

Fig. 4. Model of signal transduction in metal response. During Cd stress, the NAC complex (filled rectangles), with this particular BTF3 subunit, binds WASP nascent polypeptide chains (thick line) formed by the ribosome. The bound complex is protected from the signal recognition particle (SRP, filled oval). The intracellular location of WASP is probably the nucleus where WASP mediates signal transduction to cause cAMP activation of a protein kinase. In accordance to classical cAMP/Ca^{2+} interactions, high Ca^{2+} level reduces the level of cAMP. In JS237, which fails to form the HMW PC-CdS complex and is more sensitive to Ca^{2+}, functional BTF3 is not available to form the NAC complex. Consequently, SRP recognition of WASP directs it to the ER, resulting in the loss of signal transduction and phosphorylation of HMT1 and possibly other targets.

protein kinases, it is attractive to postulate that cAMP addition overcomes the mutation by affecting a step downstream of the signal transduction pathway. Our current thinking is that WASP must be targeted to a designated location, such as to the nucleus, to mediate signal transduction from metal stress. The signal is transduced to elevate the level of cAMP which then activates a protein kinase to phosphorylate target proteins. In the absence of the associated NAC, WASP is missorted to the ER, thus aborting the signal relay (Fig. 4).

A tempting speculation is that one of the target proteins that must be phosphorylated is HMT1. This would provide a plausible means to regulate its activity in the absence of a difference in protein levels with or without cadmium stress. The regulation of activity through phosphorylation for members of this transport family is not without precedent. The cystic fibrosis transmembrane conductance regulator which transports Cl^- ions is regulated by cAMP and phosphorylation. One observation that should be noted is that JS237 is a slow growing mutant, even in

the absence of Cd^{2+}. Thus, the mutation probably affects additional targets other than HMT1. It is possible that this mutant will lead to identifying additional targets that are activated through phosphorylation during metal stress.

2.8. A second DNA fragment that confers hypertolerance

We have recently cloned a DNA fragment that can complement JS246, a mutant that lacks detectable Cd-bound complexes. Whereas the DNA fragment that complements JS563 confers only slight hypertolerance when present on a high copy vector, the DNA that complements JS246 confers hypertolerance to both mutant and wild type cells. In this regard, it is similar to the effect shown by multiple copies of *hmt1*, which through hyperproduction of the PC-Cd transporter enhances tolerance and sequestration of cadmium. This behavior is consistent with the possibility of it encoding an enzyme in PC biosynthesis.

3. Conclusions

Research that focuses on the basic aspects of metal detoxification and accumulation is important to provide insight into the molecular details of the metal response in higher plants. Without this fundamental understanding, there will be limited opportunities for the genetic manipulation of plants for phytoremediation. Listed below are examples of how recent findings impact on strategies for the genetic engineering of plants for phytoremediation.

(1) The initial discovery and cloning of a PC-Cd transporter provided an exciting possibility for engineering high level production of this protein in higher plants. However, equally important is the uncovering of a new Cd-induced sulfide-generating pathway. Should the engineering of high capacity Cd transport succeeds, then the assembly of the stable HMW chelate would require commensurately higher sulfide levels. Maximum sequestration could therefore depend on not only enhanced metal transport but also enhanced sulfide production.

(2) The discovery that Pb induces sulfide production through the familiar assimilatory sulfate reduction pathway has provided a plausible means to hyperaccumulate Pb through manipulation of this pathway.

(3) The discovery of a FAD/NAD-linked disulfide reductase implies that increasing metal chelation cannot be achieved by simply engineering high-level production of phytochelatin peptides. In the absence of sufficient reducing power, excess peptides would become oxidized and ineffective for metal binding.

(4) Discovery of a mannose-1-phosphate guanyltransferase suggests that glycosylation may be needed for proper localization of HMT1 or some other component of the Cd-transport pathway. This implies that engineering efforts in reproducing the fission yeast Cd transport system in plants must consider plant glycosylation and sorting of foreign proteins. Given that little is known about protein sorting to the plant vacuolar membrane, this new finding has redirected focus to the need to clone the plant homologue of this gene, where signals for proper protein targeting would be present.

(5) The discovery of BTF3, WASP, cAMP, and Ca^{2+} regulation has implicated phosphorylation in the metal response, and possibly in the control of the HMT1 transporter. If phosphorylation is indeed needed for activity, efforts to engineer high level production of HMT1, its plant homologue, or other key proteins in plants will require a way to keep the additional proteins in their transport-active state. If the cell lacks sufficient kinase activity or self regulates to allow only a certain amount of the HMT1 protein in an active conformation, then increased protein production would fail to yield a linear increase in Cd transport. Since this regulatory feature is probably an evolutionary adaptation for energy conservation purposes, it may be possible to isolate mutant proteins that can remain active without phosphorylation. Toward that end, deeper understanding of this process is desired, including the phosphorylation sites that regulate activity.

To conclude, the above examples illustrate how apparently basic information can nonetheless be crucial for genetic engineering efforts. These and future findings will provide critical insight into metal and radionuclide bioremediation.

References

[1] Banuelos, G.S., Cardon, G., Mackey, B., Ben-Asher, J., Wu, L., Beuselinck, P., Akohoue, S. and Zambrzuski, S., 1993. Boron and selenium removal in B-laden soils by four sprinkler irrigated plant species. J. Environ. Qual., 22: 786–797.

[2] Zayed, A.M. and Terry, N., 1994. Selenium volatilization in roots and shoots: effects of shoot removal and sulfate level. J. Plant Physiol., 143: 8–14.

[3] Rugh, C.L., Wilde, H.D., Stack, N.M., Thompson, D.M., Summers, A.O. and Meagher, R.B., 1996. Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial *merA* gene. Proc. Natl. Acad. Sci. USA, 93: 3182–3187.

[4] Moffat, A.S., 1995. Plants proving their worth in toxic metal cleanup. Science, 269: 302–303.

[5] Baker, A.J.M. and Brooks, R.R., 1989. Terrestrial higher plants which hyperaccumulate metal elements — a review of their distribution, ecology, and phytochemistry. Biorecovery, 1: 81–126.

[6] Cunningham, S. and Ow, D.W., 1996. Promises and prospects of phytoremediation. Plant Physiol., 110: 715–719.

[7] Ow, D.W., 1993. Phytochelatin-mediated cadmium tolerance in *Schizosaccharomyces pombe*. In Vitro Cell. Dev. Biol., 29P: 213–219.

[8] Hamer, D.H., 1986. Metallothioneins. Annu. Rev. Biochem., 55: 913–951.

[9] Misra, S. and Gedamu, L., 1989. Heavy metal tolerant transgenic *Brassica napus* L. and *Nicotiana tabacum* L. plants. Theor. Appl. Genet., 78: 161–168.

[10] Yeargan, R., Maiti, I.B., Nielsen, M.T., Hunt, A.G. and Wagner, G.J., 1992. Tissue partitioning of cadmium in trangenic tobacco seedlings and field grown plants expressing the mouse metallothionein I gene. Transgenic Res., 1: 261–267.

[11] Elmayan, T. and Tepfar, M., 1994. Synthesis of a bifunctional metallothionein/beta-glucuronidase fusion protein in transgenic tobacco plants as a means of reducing leaf cadmium levels. Plant J., 6: 433–440.

[12] Hattori, J., Labbe, H. and Miki, B.L., 1994. Construction and expression of a metallothionein-beta-glucuronidase gene fusion. Genome, 37: 508–512.

[13] Pan, A., Tie, F., Duau, Z., Yang, M., Wang, Z., Li, L., Chen, Z. and Ru, B., 1994. Alpha-Domain of human metallothionein I-A can bind to metals in transgenic tobacco plants. Mol. Gen. Genet., 242: 666–674.

[14] Robinson, N.J., Tommey, A.M., Kuske, C. and Jackson, P.J., 1993. Plant metallothioneins. Biochem. J., 295: 1–10.

[15] Steffens, J.C., 1990. The heavy metal-binding peptides of plants. Annu. Rev. Plant Physiol. Plant Mol. Biol., 41: 553–575.
[16] Rauser, W., 1995. Phytochelatins and related peptides. Plant Physiol., 109: 1141–1149.
[17] Grill, E., Winnacker, E.L. and Zenk, M.H., 1987. Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. Proc. Natl. Acad. Sci. USA, 84: 439–443.
[18] Thumann, J., Grill, E., Winnacker, E.-L. and Zenk, M.H., 1991. Reactivation of metal-requiring apoenzymes by phytochelatin-metal complexes. FEBS Lett., 284: 66–69.
[19] Mehra, R.K., Kodati, V.R. and Abdullah, R., 1995. Chain length-dependent Pb(II)-coordination in phytochelatins. Biochem. Biophy. Res. Commun., 215: 730–736.
[20] Mehra, R.K., Miclat, J., Kodati, V.R., Abdullah, R., Hunter, T.C. and Muchandani, P., 1996 Optimal spectroscropic and reverse-phase HPLC analysis of Hg(II) binding to phytochelatins. Biochem. J., 314: 73–82.
[21] Maitani, T., Kubota, H., Sato, K. and Yamada, T., 1996. The composition of metals bound to class III metallothionein (phytochelatin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*. Plant Phsyiol., 110: 1145–1150.
[22] Ortiz, D.F., Kreppel, L., Speiser, D.M., Scheel, G., McDonald, G. and Ow, D.W., 1992. Heavy metal tolerance in the fission yeast requires an ATP binding cassette-type vacuolar membrane transporter. EMBO J., 11: 3491–3499.
[23] Ortiz, D.F., Ruscitti, T., McCue, K. and Ow, D.W., 1995. Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. J. Biol. Chem., 270: 4721–4728.
[24] Salt, D.E. and Rauser, W.E., 1995. MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiol., 107: 1293–1301.
[25] Salt, D.E. and Wagner, G.J., 1993. Cadmium transport across tonoplast of vesicles from oat roots. Evidence for a Cd^{2+}/H^{+} antiport activity. J. Biol. Chem., 268: 12297–12302.
[26] Stasdeit, H., Duhme, A.-K., Kneer, R., Zenk, M.H., Hermes, C. and Nolting, H.-F., 1991. Evidence for discrete Cd(SCys)4 units in cadmium phytochelatin complexes from EXAFS spectroscopy. J. Chem. Soc. Chem. Commun., 16: 1129–11130.
[27] Dameron, C.T., Reese, R.N., Mehra, R.K., Kortan, A.R., Carroll, P.J., Steigerwald, M.L., Brus, L.E. and Winge, D.R., 1989. Biosynthesis of cadmium sulphide quantum semiconductor crystallites. Nature, 338: 596–597.
[28] Reese, R.N. and Winge, D.R., 1988. Sulfide stabilization of the cadmium-γ-glutamyl peptide complex of *Schizosaccharomyces pombe*. J. Biol. Chem., 263: 12832–12835.
[29] Speiser, D.M., Ortiz, D.F., Kreppel, L., Scheel, G., McDonald, G. and Ow, D.W., 1992. Purine biosynthetic genes are required for cadmium tolerance in *Schizosaccharomyces pombe*. Mol. Cell. Biol., 12: 5301–5310.
[30] Juang, R.-H., McCue, K.F. and Ow, D.W., 1993. Two purine biosynthetic enzymes that are required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate in vitro. Arch. Biochem. Biophys., 304: 392–401.
[31] Porter, D.J.T., Rudie, N.G. and Bright, H.J., 1983. Nitro analogs of substrates for adenylosuccinate synthetase and adenylosuccinate lyase. Arch. Biochem. Biophys., 225: 157–163.
[32] Zheng, X.M., Black, D., Chambon, P. and Egly, J.M., 1990. Sequencing and expression of complementary DNA for the general transcription factor BTF3. Nature, 344: 556–559.
[33] Wiedmann, B., Sakai, H., Davis, T.A. and Wiedmann, M., 1994. A protein complex required for signal-sequence-specific sorting and translocation. Nature, 370: 434–440.
[34] Derry, J.M., Ochs, H.D. and Francke, U., 1994. Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. Cell, 78: 635–644.
[35] Rivero-Lezcano, O.M., Marcilla, A., Sameshima, J.H. and Robbins, K.C., 1995. Wiskott-Aldrich syndrome protein physically associates with Nck through Src homology 3 domains. Mol. Cell. Biol., 15: 5725–5731.